Several Log Increase in Therapeutic Transgene Delivery by Distinct Adeno-Associated Viral Serotype Vectors

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We previously demonstrated that rAAV vectors carrying human and canine factor IX (FIX) cDNA can infect, stably persist, and secrete functional human and canine FIX following direct intramuscular injection. In an attempt to improve FIX protein secretion for eventual therapeutic use, we set out to determine if alteration of the AAV capsid would affect skeletal muscle transduction and factor IX secretion. Two reasons to pursue this question were (1) the persistence of high-titer neutralizing antibody (NAB) to AAV2 and (2) our previous study that supported a restricted tropism of muscle fiber types to AAV2 transduction. Using an identical CMV/canine factor IX (cFIX) expression cassette, we cross-packaged this genome into virions generated from each of the five AAV serotypes. In a dose–response assay, equivalent amounts of rAAV/cFIX serotypes were tested *in vitro* and *in vivo*. In tissue culture cells, FIX antigen levels secreted into the supernatant varied depending on the AAV serotype used; type 2 transduced maximally, with serotypes 3, 1, 5, and 4, respectively, expressing lower levels. However, when the same viruses were tested *in vivo* using immunodeficient NOD/SCID animals, we obtained surprisingly different results. While the time to onset of detectable serum levels appeared the same for all serotypes, types 1, 3, and 5 produced 100-to 1000-fold more cFIX than type 2. In fact, 12 weeks after transduction, type 1 continued to express levels of cFIX on average at 80 µg/ml followed by type 5 (6.52 µg/ml), type 3 (3.27 µg/ml), type 4 (258 ng/ml), and finally type 2 (90 ng/ml). Coagulant activity of cFIX as measured by a PTT supported the circulating levels measured by ELISA demonstrating the secreted protein was functional, and RT-PCR of injected tissue correlated with the serotype-specific transduction data. In summary, we found significant differences in cFIX expression upon introducing various rAAV serotypes into mouse muscle. These data have direct bearing on the design of AAV gene therapy clinical trials for hemophilia and should also extend to most therapeutic transgenes.

INTRODUCTION

Recombinant adeno-associated virus (rAAV) is considered a safe and promising vector for human gene therapy. AAV is nonpathogenic, infects both dividing and nondividing cells, can establish latency, and persists in tissue such as skeletal muscle, retina, liver, brain, and vasculature (1). A variety of therapeutic proteins including factor IX (FIX) are expressed via simple intramuscular injection of recombinant AAV (2–4). Of the six AAV serotypes, serotype type 2 (AAV2) is best-characterized and therefore predominantly used in gene transfer studies (5). Here we report that AAV vectors types 1, 3, 4, and 5 transduce skeletal muscle more efficiently and secrete canine

FIX (cFIX) at levels two-to-three logs greater than rAAV2. Thus, without modifying gene expression cassettes, the capsid composition of the majority of AAV serotype appears to effect vector transduction significantly enough to produce higher than therapeutic levels of circulating FIX.

Six serotypes of AAV have been cloned and sequenced with five of the six having divergent amino acid sequences. Serotypes 1 and 6 share >99% amino acid homology in their capsid proteins and sequence analysis supports a recombination event between serotypes 1 and 2 (6, 7, 14). Comparison of the serotype capsid amino acid sequences suggests that types 1, 2, and 3 share homology across the three capsids in accord with heparan sulfate binding (8). In contrast, AAV types 4 and 5 share less homology across the capsid region and neither type 4 nor type 5 binding is competed by soluble heparan sulfate, suggesting that they use distinct receptors (9, 10).

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Hemophilia B is the clinical manifestation of inherited factor IX deficiency (11). We and others demonstrated that AAV2 targeting skeletal muscle produces functional factor IX in large animal models but at less than therapeutic levels (2, 12). Recent reports in brain suggest that AAV serotypes 4 and 5 have different host cell tropisms *in vivo* (13). With respect to skeletal muscle transduction, AAV2 preferentially infects slow muscle fiber groups, indicating a restricted tropism in muscle (15), whereas type 1 has been shown to have a marked increase in muscle transduction (14).

METHODS

rAAV production. All rAAV vectors were produced, purified, and titered as previously described (2). The rAAV/green fluorescence protein (GFP) or cFIX DNA template sutilized for encapsidation in AAV1,2,3, and 4 were derived from plasmid psub201 as previously described (2). rAAV5 virus was generated after subcloning the EGFP and cFIX cDNAs into plasmid p7D05 containing two type 5 ITRs (10) via *Bgl*II digestion. The AAV5 vector plasmid p7D05 and helper plasmid pAAV5 were generously supplied by Drs. Chiorini and Kotin (NHLBI, NIH, Bethesda, MD). All other AAV packaging plasmids were supplied through the UNC Gene Therapy Vector Core and described by Rabinowitz and Samulski (submitted).

Canine FIX assays. Canine FIX antigen and activity assays (ELISA and aPTT) were performed as previously described (2) with minor modifications. Purified canine FIX (Affinity Biological Inc., Hamilton, Canada) was added to pooled NOD/SCID mice plasma and serial dilutions were made. This was used as standard for ELISA and aPTT. Plasma from experimental animals was diluted 4- to 400-fold for both ELISA and aPTT assays. Plasma from animals that were injected with rAAV/EGFP was used as control.

Animal care and procedures. NOD/SCID animals (Jackson Labs, Bar Harbor, ME) were maintained and treated in accordance with the Animal Care and Use Committee of UNC Chapel Hill. One hundred microliters of PBS containing 1.5 × 10^{10} rAAV/GFP or 2.5 × 10^{11} rAAV/cFIX virions was directly injected into the gastrocnemius muscle in 4- to 5-week-old NOD/SCID mice. The mice were bleed via retroorbital plexus every 4 weeks postinjection. Eight weeks after rAAV injections, the mice receiving rAAV/GFP were anesthetized by 2.5% avertin and perfused through cardiac puncture with 4% paraformaldehyde (Sigma) and 0.25% glutaraldehyde (Sigma). The gastrocnemius muscles with injected rAAV (marked with coinjection of carbon particles) were harvested and sliced at 25- μm intervals. The muscle specimens were observed by fluorescent microscopy (Model DM-IRB, Leica, Germany). Twelve weeks after rAAV injection, mice in each experimental group receiving serotyped rAAV/cFIX were sacrificed, and the skeletal muscle was harvested and processed. RNA was isolated using a Qiagen Rneasy kit (Qiagen, Germany), following the manufacturer's protocol. RT-PCR was performed as described previously (2).

RESULTS

To investigate all the AAV serotypes for efficient muscle transduction, we generated high-titer rAAV type 1, 2, 3, 4, and 5 viruses expressing humanized GFP or canine factor IX by transient triple-plasmid transfection as previously described (2). An AAV type 2 vector plasmid carrying either a CMV-driven EGFP or cFIX gene was used with the appropriate helper plasmid (see Methods) to cross-package and generate rAAV/EGFP or rAAVcFIX 1, 2, 3, and 4 vectors. These recombinant vectors differ only in capsid structure (Rabinowitz and Samulski, submit-
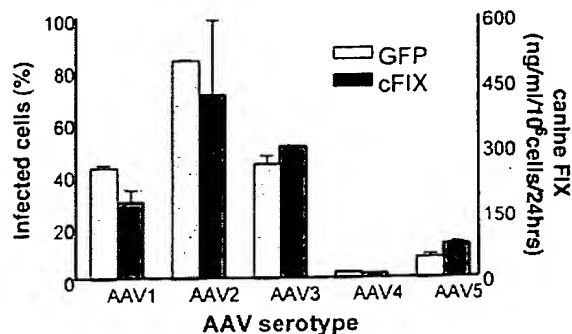

FIG. 1. 293 cells were infected with either rAAV1-to-5/GFP or rAAV1-to-5/cFIX at a m.o.i. of 10^3 vector genomes/cell. Quantitation of each virus preparation (vector genomes/ml) by dot-blot hybridization was performed prior to each experiment and all vectors were prepared, stored, and used identically. Adenovirus type 5 (wt ad5) was added at a m.o.i. of 2/cell, and rAAV1-to-5/GFP-infected cells were analyzed by flow cytometry 24 h postinfection. The percentage of GFP-positive cells was determined, and cFIX antigen in the cell supernatant was measured by ELISA. No detectable fluorescence or cFIX was observed in mock-infected controls (Ad5 only, data not shown).

ted). To generate rAAV5, we subcloned the EGFP or cFIX expression cassette in to p7D05 that contains rAAV5 ITR elements. This vector was cotransfected with pAAV5 (containing the rep and cap genes of AAV5) as previously described (10) to generate rAAV5/EGFP and rAAV5/cFIX. All viruses were purified and particle numbers determined by dot-blot assay more than three times.

Serotype-Specific Transduction in Vitro

Each recombinant serotype was tested for transduction by infecting the human-derived 293 cell line in duplicate using identical numbers of viral particles. Serotype 2 EGFP and cFIX vectors produced the highest transduction levels (Fig. 1). These observations were followed by AAV types 3, 1, and 5 and finally serotype 4. The low level of rAAV4/EGFP fluorescence and rAAV4/cFIX levels secreted were consistent with previous reports demonstrating the inefficient transduction of human vs nonhuman primate cells (9). In addition, the difference observed with type 2 transduction on 293 cells may be related to the fact that this cell has been characterized for expressing cell-surface heparan sulfate which is employed by AAV2 as one of its cell receptors (10). At present, none of the receptor molecules used by the other serotypes has been described. In these studies, we used vector particle number to control for m.o.i. Because both GFP and cFIX vector preps transduced equally for each respective serotype (Fig. 1), we concluded that transduction differences observed between serotypes were not due to variation from vector production and purification. To confirm these observations, specific serotypes were produced more than once and retested for *in vitro* and *in vivo* transduction with similar results.

FIG.2. *Invivo* transductionofskeletalmuscleusingrAAVserotypes. rAAV/EGFPtypes1–5wereinjectedintothehindlimbsof4-week-oldNOD/SCIDmiceat 1.5 × 10^{10} genomes/animal,respectively.Miceweresacrificed8weekspostinjection;theinjectedskeletalmusclewasprocessedandphotog raphed(magnification200 x).(A)rAAV1/GFP-injectedmuscle,(B)rAAV2/GFP-injectedmuscle,(C)rAAV5/GFP-injectedmuscle.Tocontrolforbackgroundfluorescence,a500-nmband-widthpassfilterwasused.

Serotype-SpecificTransductioninVivo

Direct intramuscular injection of rAAV2 transduces skeletal muscle cells and persists long-term in animal models(2).Toinvestigate*invivo* transductionefficiency, thefivedifferentserotypesofrAAV/GFPandcFIXwere directly injected into gastrocnemius muscle of 4- to 5-week-oldNOD/SCIDmice(seeMethods).Skeletalmuscle fluorescence for GFP transduction measured at 8 weeks was positive for all five rAAV serotypes with AAV1 and AAV5displayingthemostintenseandwidespreadstaining.TheGFPstainingpatternsuggeststhattype1and5 vectors diffused across the length of the entire muscle analyzed unlike the "patchwork" appearance typically observedwithAAV2(Fig.2).
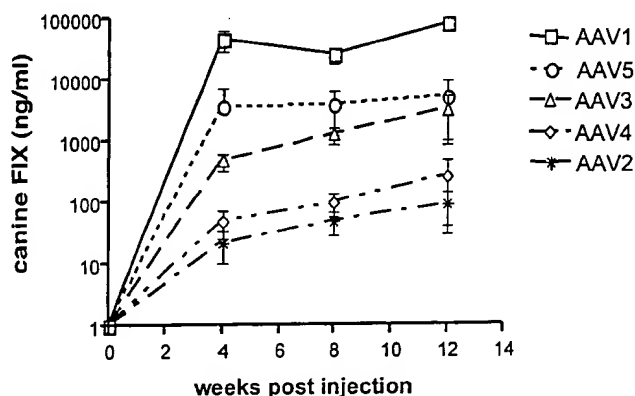

FIG.3. PlasmaantigenlevelsofcanineFIXinNOD/SCIDmiceafterintramuscular injection of rAAV serotypes. Mice received 2.5 × 10^{11} vector genomesofeachAAV/cFIXserotype.Avolumeof100 µlviruswasinjected intothegastrocnemiusmuscle(*n* =5miceforeachserotypetested).Mice werebledatmonthlyintervalsandtheplasmawasmeasuredbyELISAfor cFIXantigen.Plasmafromanimalsinjectedwith AAV/EGFPwasusedascontrol.

Based on these results, we then tested the five serotypeAAV/canineFIXvectorsinskeletalmuscle.2.5 × 10^{11} genomeparticlesofrAAV/cFIX1,2,3,4,and5 wereinjectedintothehindlimbsofNOD/SCIDmice(*n* =5foreachexperimentalgroup).Plasmawastestedat monthly intervals for canine factor IX using an ELISA specificforcaninefactorIX(seeMethods).Asshownin Fig. 3, wedetectedcaninefactorIXinallexperimental animals.NocaninefactorIXantigenwasdetectedfrom plasma samples taken from control animals receiving equivalent doses of serotyped AAV/EGFP (data not shown).Asexpectedandconsistentwiththe GFPstudies,cFIXantigenlevelsinmicereceivingrAAVtypes1,3, 4,and5werehigherthanthoseintherAAV2/cFIXmice (Fig.3).TheamountofcaninefactorIXintheplasmaof micereceivingtypes1,3,and5wasunexpected.Levels higherthan100and10 µg/mlofcFIXweredetectedin themicereceivingrAAVtypes1and5,respectively.Even serotype3,whichbysequenceismostsimilartotype2, expressed cFIX levels up to 5.8 µg/ml. This represents 10^3 and10^2 increasesofcFIX,respectively,whencomparedtomicereceivingrAAV2/cFIX.

Vector-TransducedCanineFIXIsBiologicallyActive

The supraphysiologic levels of cFIX allowed us to dilutetheendogenousmouseFIXandtestthecoagulant activityoftransgeneprotein.TheaPTTof(normal)control mice was significantly prolonged at 5-fold and greaterdilutionsofplasma(Table1).Inmarkedcontrast, 80- to 160-fold dilutions of rAAV1/cFIX mouse plasma were required before the aPTT time was prolonged. To quantifytheaPTTactivityintermsofabsoluteamounts ofprotein,weestablishedastandardaPTTcurvebythe addition of purified canine factor IX to control mouse plasma (data not shown). Based on these results, cFIX antigenandactivitylevelswereconsistentandindicated

TABLE1
aPTTClottingTimesofthePlasmaofrAAV1/cFIX-TreatedMice

| Dilution factor | Control plasma | Clottingtime(s) | | | | | PurifiedcFIX inmiceplasma |
| | | AAV1/cFIXmice | | | | | |
		1629	1630	1885	1886	1887	
1:5	96.6	ND	ND	ND	ND	ND	—
1:10	113.1	51.3	ND	ND	ND	ND	—
1:20	ND	71.9	65.1	ND	ND	ND	86.1(900ng/ml)
1:40	ND	84.8	80.9	79.9	88.4	82.6	95.6(450ng/ml)
1:80	ND	99.15	94.7	89.6	108.9	104.4	105.1(225ng/ml)
1:160	ND	137.3	113.1	109.6	122.6	123.1	114.9(112.5ng/ml)

Note. Plasma was collected using heparin-coated microhemocrit capillary (Fisher); heparin was inactivated using Dade Hepzyme (Dade Behring Marburg Gmbh, Germany) following the manufacturer's protocol. Plasma from the rAAV1/GFP-treated mouse (control plasma) was diluted 5-to 10-fold for aPTT assay. Plasma from the rAAV1/cFIX-treated mice was diluted 10- to 160-fold. Purified cFIX was added to control plasma and diluted serially. All results are expressed as averages of duplicate samples performed in two separate experiments.

that the canine factor IX secreted was fully functional (Fig.4).

RT-PCRnalysisofVector-TransducedMuscle

Molecular analysis of rAAV/cFIX mRNA in mouse skeletal muscle was performed from each serotype-specific transduced group. At 12 weeks postinjection, animalsweresacrificed,gastrocnemiusmusclewasisolated, and mRNA was prepared for RT-PCR. Based on semi-quantitativePCR(Fig.5),theamountofdetectabletranscriptwasconsistentwiththeplasmalevelsoffactorIX (Fig. 3) and the extent of vector spread observed with GFPexpression(Fig.2).

DISCUSSION

Inthisstudy,wecharacterizedthetransductionofallfive AAVserotypesasvectorscarryinganidenticalexpression cassetteforGFPandcFIXtransgenes.Allserotype-specificvectorsproducedsimilarvectoryields(10^{12}–10^{13} particles/ml)andwerepositivefortransductionboth*invitro* and *invivo*. However,leveloftransduction *invivo* didnot mirror *invitro* results.Infact,weobserved *invivo* expression levels of cFIX that were 100 to 1000 times higher than the commonly used serotype 2 vectors. The cFIX protein expressed from all vectors was biologically active based on aPTT assays, and RT-PCR supported serotype-specific transduction levels. Based on our studies, AAV type 1 vectorsappearedtobethemostefficientinmuscletransduction,withexpressionlevelsofcFIXthataveraged 80 µg/ml followed by type 5 (6.52 µg/m), type 3 (3.27 µg/ml),type4(258ng/ml),andfinallytype2(90 ng/ml). A previous report comparing AAV type 1 and AAV type 2 vectors in muscle using different reporter genesandbreedsofmice(14)alsodescribedanincreased vectortransductionfortype1albeitatlowerlevels.

One primary reason for investigating different AAV serotype vectors was aimed at evaded existing immune responses to AAV serotype 2. Antibody to AAV2 in the normalpopulationrangesbetween50and96%.Ofthese individuals, 18–67% carry neutralizing antibodies. We havefoundinbothmiceanddogsthatneutralizinganti-bodiesarelonglastingandremainathightiterafterpri-maryexposuretoAAV2,makingreadministrationofthis serotype ineffective (Chao and Walsh, unpublished data). From these animal studies, the existing immune responsetotype2vectormayhaveasignificantimpact on *in vivo* gene transfer in humans. The lack of cross-reactivity among neutralizing antibodies of different rAAV serotypes in mice (Li and Samulski, unpublished data) suggests that vector readministration using non-AAV type 2 vectors may bypass this problem. Although wefeelconfidentthatthedifferentserotypevectorswill provide alternative approaches to readministration, moreimportantisthefactthatweobtainedhigherthan therapeuticlevelsofFIXusingnon-type2AAVvectors.
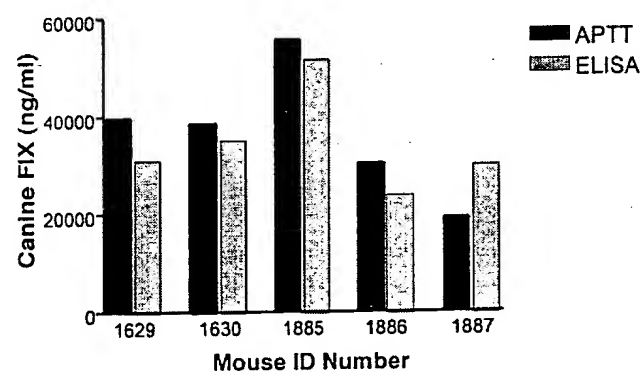

FIG. 4. Procoagulant activity of canine FIX in serotype 1-treated animals. ELISA antigen and aPTT activities were measured in plasma samples from AAV1-treatedanimals.Resultsareexpressedasaveragesofsamplesperformed in duplicate for each animal tested. Purified cFIX was added to NOD/SCID mouseplasmaandserialdilutionsweremadetogenerateastandardcurveof aPTT activity. Plasma samples of rAAV1-treated animals were diluted 10- to 160-fold. cFIX values (ng/ml) were calculated based on the aPTT standard curve. aPTT activity of plasma from AAV/EGFP-injected animals was used as control.
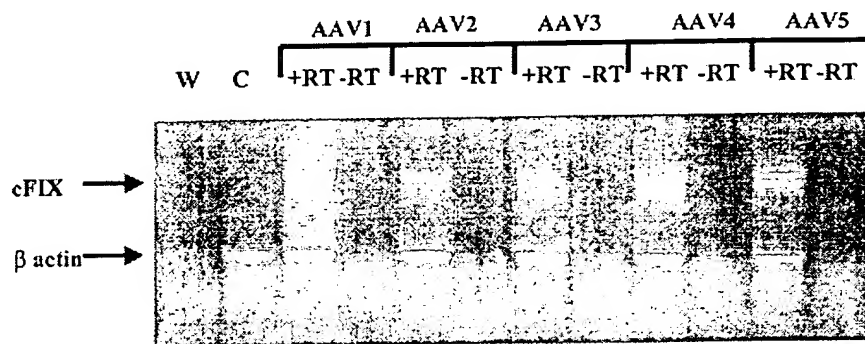
FIG.5. RNAexpressionofAAV/cFIXtypes1–5inskeletalmuscle.TotalcellularRNAwasisolatedfromskeletalmuscleandRT-PCRperformedasdescribed underMethods.PrimersspecificforthecFIXtransgeneamplifieda1000-bpband. β-Actin(250-bpband)wasusedasacontrol.RNAsamplesweretreatedwith DNase;eachsamplewastreatedwith(+RT)andwithoutreversetranscriptase(−RT)tocontrolforDNAcontamination.Water(W)andRNAisolatedfromcontrolanimalmuscle(C)areshownattheleft.Eighthundrednanogramsof RNAwasusedineachRT-PCR.

To our knowledge, this is the first report obtaining significantly higher than physiological levels of cFIX expressionwithoutmodifyingtheexpressioncassette.In fact,theuseofidenticalexpressioncassettesinthesevariousserotypesstronglysupportsviraltropismastheprimaryreasonforincreasedtransduction.Thesedatawere supported by serotype-specific GFP transduction in mousemuscle.Atpresent,allanimalstudiesusingAAV vectors have consistently yielded long-term transgene expression. In our hands, we have positive expression approaching3yearsinthehemophilicdogmodel.This strongly suggests that repeated administration may not be necessary if therapeutic levels can be obtained with initialdoses.Inaddition,ourdatainthismousemodel stronglysupportusingserotype1AAVvectorsforhuman clinical trials. One concern with testing these serotype-specificvectorsinmousemodelsisthattheseresultsmay bespecies-specific.However,thishasnotbeenthecase with serotype 2 vectors, which have successfully transducedmice,rabbit,dog,andmonkey.Atpresent,weare testing large-animal models to determine if these serotype-specificvectorswillperformequivalentlyinall models(mouse,dog,andprimate).

Finally,theresultsofthisstudysuggestthatAAVvector-mediatedgenetransferissubstantiallyimprovedby using naturally occurring serotypes as vectors. This approachmaybeusefulforthetreatmentofawidevarietyofinheriteddiseases.

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